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Effect of lecithin:cholesterol acyltransferase on distribution of apolipoprotein A-IV among lipoproteins of human plasma

Charles L. Bisgaier,[1] Om P. Sachdev, Elsie S. Lee, Kevin J. Williams, Conrad B. Blum, and Robert M. Glickman

Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032

Abstract The effect of cholesterol esterification on the distribution of apoA-IV in human plasma was investigated. Human plasma was incubated in the presence or absence of the lecithin:cholesterol acyltransferase (LCAT) inhibitor 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and immediately fractionated by 6% agarose column chromatography. Fractions were monitored for apoA-IV, apoE, and apoA-I by radioimmunoassay (RIA). Incubation resulted in an elevated plasma concentration of cholesteryl ester and in an altered distribution of apoA-IV. After incubation apoA-IV eluted in the ordinarily apoA-IV-poor fractions of plasma that contain small VLDL particles, LDL, and HDL_2. Inclusion of DTNB during the incubation resulted in some enlargement of HDL; however, both cholesterol esterification and lipoprotein binding of apoA-IV were inhibited. Addition of DTNB to plasma after incubation and prior to gel filtration had no effect on the apoA-IV distribution when the lipoproteins were immediately fractionated. Fasting plasma apoE was distributed in two or three peaks; in some plasmas there was a small peak that eluted with the column void volume, and, in all plasmas, there were larger peaks that eluted with the VLDL-LDL region and HDL_2. Incubation resulted in displacement of HDL apoE to larger lipoproteins and this effect was observed in the presence or absence of DTNB. ApoA-I was distributed in a single broad peak that eluted in the region of HDL and the gel-filtered distribution was unaffected by incubation either in the presence or absence of DTNB. Incubation of plasma that was previously heated to 56°C to inactivate LCAT resulted in no additional movement of apoA-IV onto lipoproteins, unless purified LCAT was present during incubation. The addition of heat-inactivated LCAT to the incubation, had no effect on movement of apoA-IV. These data suggest that human apoA-IV redistribution from the lipoprotein-free fraction to lipoprotein particles appears to be dependent on LCAT action. The mechanism responsible for the increased binding of apoA-IV to the surface of lipoproteins when LCAT acts may involve the generation of "gaps" in the lipoprotein surface due to the consumption of substrate from the surface and additional enlargement of the core. ApoA-IV may bind to these "gaps," where the packing density of the phospholipid head groups is reduced.—**Bisgaier, C. L., O. P. Sachdev, E. S. Lee, K. J. Williams, C. B. Blum, and R. M. Glickman.** Effect of lecithin:cholesterol acyltransferase on distribution of apolipoprotein A-IV among lipoproteins of human plasma. *J. Lipid Res.* 1987. **28:** 693-703.

Supplementary key words HDL • LDL • VLDL • apoA-I • apoE • gel filtration of plasma

Apolipoprotein A-IV (apoA-IV) is a 46,000 molecular weight protein (1) synthesized by both liver and intestine (2-7). Recent evidence in humans suggests that expression of apoA-IV mRNA is largely restricted to the intestine, while rat message is made by both liver and intestine (8, 9). The 396 amino acid sequence of human preapoA-IV, which includes a 20 amino acid "pre" segment, has been deduced from its cDNA (9, 10); however, there are discrepancies in the reported sequences coding for amino acids 138, 140, 259, 307, and 360 (9, 10). With fat-feeding apoA-IV is secreted on chylomicrons. However, only small amounts of apoA-IV can be found on triglyceride-rich lipoproteins in plasma (11, 12) since the apolipoprotein is rapidly displaced by C apolipoproteins and apoE (13, 14). Postprandial lipemic plasma has an elevated concentration of apoA-IV (11, 12); however, the distribution of apoA-IV among lipoproteins is similar to that of fasting plasma. In the fasting human, small amounts of apoA-IV are associated with the major HDL fraction of plasma while most apoA-IV appears to exist unassociated with the major lipoproteins (11, 12, 15-18). Ultracentrifugation of human lipoproteins removes most apoA-IV from them (11, 12). However, using noncentrifugal techniques, the presence of apoA-IV on human HDL has been demonstrated by

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Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; PBSA, 1% bovine serum albumin and 0.02% sodium azide in 50 mM phosphate-buffered saline.

[1]Address reprint requests to Charles Bisgaier, Dept. of Medicine, GI Division, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032.

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gel filtration of plasma (11, 12, 15), apoA-IV affinity chromatography of HDL (16), and immunoprecipitation of gel-filtered plasma HDL with anti-apoA-I serum (12). Recent studies of DeLamatre et al. (19) have shown that apoA-IV in gel-filtered plasma of the fasting rat is equally distributed between lipoproteins (mainly HDL) and lipoprotein-free fractions. These investigators demonstrated that incubation of rat plasma resulted in esterification of cholesterol and a marked redistribution of apoA-IV from the lipoprotein-free fraction to lipoprotein-containing fractions. Inhibition of LCAT activity by DTNB prevented this redistribution of apoA-IV. In the same experiments the distribution of rat apoA-I and apoE were essentially unaffected, suggesting some specificity for the relationship between cholesterol esterification and apoA-IV distribution. Although the physiologic function of apoA-IV is unknown, studies of the activation of LCAT by apolipoproteins using artificial phospholipid cholesterol apolipoprotein substrates suggest that apoA-IV may play a cofactor role in facilitating the LCAT reaction (20). When saturated phosphatidylcholines were used as substrate for the LCAT reaction, apoA-IV was always a better activator than apoA-I (20). While physicochemical characterization of apoA-IV isolated from human and rat show conformational similarities (21), they are immunologically distinct (22). About half of rat plasma apoA-IV is associated with HDL, of which HDL_2 is the major subclass in this species. Human plasma predominantly contains HDL_3, which is significantly smaller than rat HDL and contains much less apoA-IV (11, 12, 15, 19). The level of the major HDL apolipoprotein, apoA-I, is normally 2.5-3 times greater in human plasma (100-120 mg/dl) (23, 24) than in rat plasma (40-50 mg/dl) (25, 26). A wide range of values for apoA-IV levels has been reported in both species (11-13, 17, 27-29). In general, the values reported for humans (13-37 mg/dl) are higher than those reported in the rat (10-24 mg/dl). Thus, in addition to the amount of lipoprotein surface available, the amount of apoA-IV relative to apoA-I and other apolipoproteins may also affect the amount of apoA-IV that is lipoprotein bound.

Because of the major differences in the way apoA-IV is associated with lipoproteins in human and rat plasma, we were concerned that experiments in the rat might not be relevant to potential roles of apoA-IV in human lipoprotein metabolism. The present experiments on the effects of LCAT activity on the lipoprotein distribution of apoA-IV in human plasma were performed in an attempt to gain insights into potential roles of apoA-IV in human lipoprotein metabolism. Thus, in the present report, we present data that demonstrate LCAT activity results in modifications to lipoprotein size, composition of cholesteryl ester, and particularly to the distribution of human apoA-IV.

MATERIALS AND METHODS

Isolation of plasma and lipoprotein fractions

Blood of fasting (12 hr) humans, drawn from an antecubital vein, was collected in tubes containing EDTA (1 mg/ml blood), and plasma was prepared by low speed centrifugation. In some experiments, following incubation of whole plasma, d 1.006-1.25 g/ml fractions were prepared by ultracentrifugation. These d 1.006-1.25 g/ml fractions were then analyzed for apoA-I distribution following lipoprotein fractionation by isopycnic density gradient centrifugation or by immunoblot analysis on 4-30% nondenaturing gradient gels (12, 30). For isopycnic density gradient centrifugation, the d 1.006-1.25 g/ml fraction was dialyzed against a NaBr solution of d 1.15 g/ml, and a 1-ml aliquot was carefully layered above 0.8 ml of NaBr solutions of d 1.25 g/ml and 1.20 g/ml, and below solutions of d 1.10 g/ml, 1.05 g/ml, and 1.00 g/ml (H_2O) in a 5.2-ml tube. Tubes were centrifuged at 50,000 rpm for 72 hr at 4°C in an SW 50.1 rotor. Seventeen fractions of approximately 300 μl each were removed from each tube with the aid of a thin elongated Pasteur pipet and analyzed for density by refractometry and apoA-I content by RIA. The d 1.006-1.25 g/ml fractions were analyzed by polyacrylamide gradient gel electrophoresis; the gels were stained for protein with Coomassie Blue G-250 (30) and for lipid with Sudan Black B (31); localization of apoA-I was by immunoblot analysis as previously described (12).

Immunological quantitation of apolipoproteins

Human apoA-IV and apoE were quantitated by RIA as previously described (12, 32). Human apoA-I was also quantitated by RIA. ApoA-I was isolated by Sephadex G-200 fractionation of delipidated human HDL (23) in 6 M urea in 10 mM Tris-HCl, pH 8.2. Antibodies to human apoA-I were raised in rabbits (33). ApoA-I was iodinated by the chloramine-T procedure (34), as previously described for apoA-IV (12), and routinely resulted in 0.3 mol of ^{125}I incorporated per mol of apoA-I. Antiserum was diluted to bind 50% of ^{125}I-labeled apoA-I and used to develop a radioimmunoassay. ApoA-I standard was used to calibrate whole or delipidated plasma. Whole plasma was used in all subsequent assays as standard. ApoA-I standard or plasma dilutions were incubated in 50 mM phosphate-buffered saline, pH 7.5, with 1% bovine serum albumin (PBSA) and 1% sodium dodecyl sulfate (SDS) for 30 min at 37°C prior to assay. ApoA-I RIA was carried out in 12 × 75 mm glass borosilicate tubes to which 100 μl of ^{125}I-labeled apoA-I (30,000 cpm in PBSA), 100 μl of nonimmune rabbit serum (1:500 stock in PBSA), 100 μl of apoA-I standard, standard plasma, or unknown sample (in 1% SDS in PBSA), and 100 μl of

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rabbit-anti-apoA-I (1:500 stock in PBSA) were sequentially added. Following a 48-hr incubation at 4°C, 100 μl of goat anti-rabbit IgG serum (1:30 stock in PBSA) was added and incubation was continued for an additional 24 hr. Immunoprecipitates were harvested by centrifugation at 3000 rpm and 30 min in a Sorvall RC-2 centrifuge. Following an additional 0.5-ml PBSA wash of the precipitates, triplicate tubes of each sample were counted in an LKB 1270 Rackgamma II gamma counter. ^{125}I-labeled apoA-I was displaced to the same extent by either whole or delipidated SDS-treated plasma, resulting in curves that were superimposable on each other and parallel to the displacement curve with authentic SDS-treated apoA-I. The linear displacement region of the assay was between 1–160 ng of apoA-I. For the apoA-I RIA, the coefficients of variation were 5.5% (intra-assay, n = 4) and 4.8% (inter-assay, n = 9). Plasma standard calibrated to 128 mg/dl in the present study and was determined to be 124 mg/dl by an independently developed apoA-I RIA (35).

Cholesterol analysis

Total and free cholesterol mass were determined by measurement of the enzymatic production of hydrogen peroxide (36) when plasma aliquots (50 μl) were incubated with cholesterol oxidase in the absence (free cholesterol) or presence (total cholesterol) of cholesterol esterase. Esterified cholesterol was calculated as the difference. In other studies, cholesterol esterification was monitored by prelabeling whole plasma at 4°C with [4-^{14}C]cholesterol (37). At intervals, aliquots (50 μl) were removed and extracted with 1 ml of acetone–ethanol 1:1 (v/v) containing nonradioactive carrier cholesterol (1 μg/μl) and cholesterol oleate (1 μg/μl). [4-^{14}C]cholesterol was separated from [4-^{14}C]cholesteryl ester by thin-layer chromatography on silica gel in a solvent system of hexane–diethyl ether–acetic acid 80:16:4 (v/v/v). Cholesterol esterification was determined by liquid scintillation counting.

Plasma incubations and gel filtration

Fresh human plasma samples were incubated for 6 hr at 37°C in the presence and absence of 1 mM DTNB. At the conclusion of the incubation, 1-ml aliquots were promptly gel-filtered. DTNB was added to unincubated plasmas or plasma incubated in its absence immediately prior to gel filtration. Additional incubations of human plasma at 37°C for 6 hr were carried out in the absence of DTNB. From these, 1-ml aliquots were immediately gel-filtered in the absence and presence of DTNB, or stored for 48 hr at 4°C in the absence and presence of DTNB prior to gel filtration with no further additions. Gel filtration was carried out at 4°C on matched 100 × 0.9 cm 6% agarose columns (Bio-Gel A-5m, 200–400 mesh), equilibrated with 154 mM NaCl, 0.01% EDTA, and 0.01% sodium azide, pH 7.0. Fractions of 1.7–2.0 ml were collected at 15-min intervals. In any single study, equal size fractions were collected from all columns. Columns were calibrated with human LDL (d 1.020–1.050 g/ml), human HDL (d 1.063–1.21 g/ml), and bovine serum albumin.

In other studies, [4-^{14}C]cholesterol-labeled plasma or unlabeled plasma was incubated at 56°C for 30 min to inactivate LCAT (19, 37). These were then incubated in the absence of LCAT or the presence of heated purified LCAT (inactive) or unheated purified LCAT (active) at 37°C for up to 24 hr and either assayed to determine the amount of [4-^{14}C]cholesterol esterified, or gel-filtered for determination of apoA-IV distribution as indicated for individual studies below.

Purification of LCAT

Human LCAT was purified by a combination of methods described by Pattnaik et al. (38), Chen and Albers (39), and Doi and Nishida (40). To purify LCAT, a d > 1.21 g/ml fraction was isolated from three units of fresh blood bank plasma. This fraction was passed over a phenyl Sepharose column (25 × 5.0 cm) pre-equilibrated with 4 M NaCl. The column was washed with normal saline until protein was no longer detected and LCAT activity was then eluted with water (38). This fraction was acidified by the addition of Na acetate (final concentration, 50 mM), pH 4.5. Precipitated proteins were discarded and the supernatant was applied to a CM52 column (40 × 2.5 cm), equilibrated with 50 mM Na acetate, pH 4.5 (38). The column void was concentrated in a 200-ml Amicon concentration cell equipped with a YM 5 membrane; the buffer was repeatedly replaced with 1 mM phosphate, pH 7.1. This fraction (20 ml) was pumped at 20 ml/hr onto an Affi-gel blue column (39) (30 × 0.9 cm) and eluted from a hydroxylapatite (30 × 2.5 cm) (39, 40) column connected in series. Fractions of 6 ml were collected. LCAT activity was monitored by the proteoliposome assay as described by Chen and Albers (41). Fractions containing LCAT were aliquoted and frozen at −70°C and individually thawed on the day of studies. Marked loss of LCAT activity was noted with prolonged storage of fractions at 4°C. Column fractions used in experiments were purified 25,000- to 30,000-fold and demonstrated a single band of 66,000–68,000 Da on SDS-polyacrylamide gels (42).

Statistical analysis

The *t*-test for paired difference was used to analyze data (43). *P* values of less than 0.05 were considered significant.

Protein determination

Protein was estimated by the method of Lowry et al. (44) using bovine serum albumin as a standard.

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RESULTS

The apoA-IV, apoA-I, and apoE distributions of unincubated gel-filtered plasmas from four normal male subjects are shown in **Fig. 1** (panel A). In fasting plasma, 1.3 ± 0.7% apoA-IV was associated with VLDL + LDL-containing fractions, 4.8 ± 1.7% was associated with HDL-containing fractions, and the remainder (93.8 ± 2.3%)

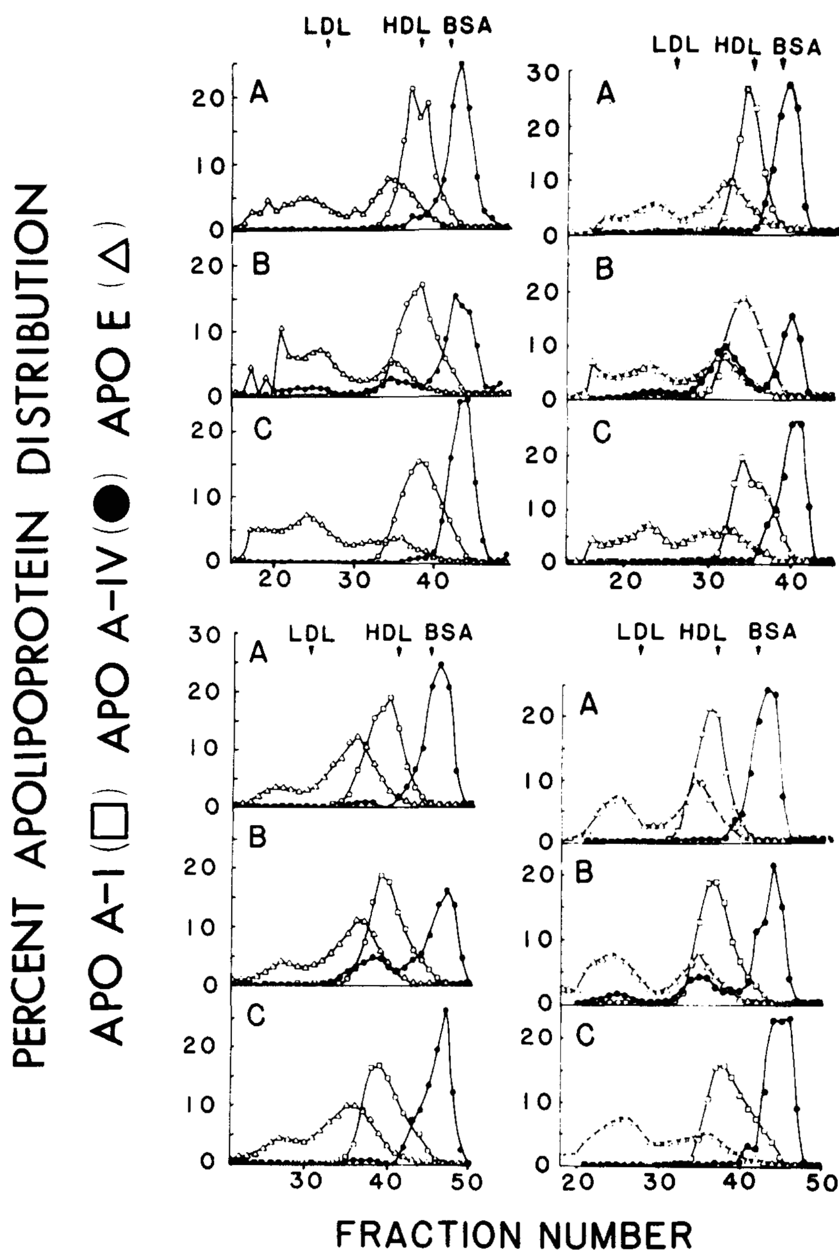

Fig. 1. ApoA-I, apoA-IV, and apoE distribution in human plasma following incubation in the absence or presence of DTNB. Unincubated human plasma (A) or plasma incubated for 6 hr at 37°C in the absence (B) or presence (C) of 1 mM DTNB were fractionated by Bio-Gel A-5m chromatography (6% agarose). An equivalent amount of DTNB was added to all plasma samples incubated in its absence immediately prior to gel filtration. Fractions were monitored for apoA-I (□), apoA-IV (●), and apoE (△) by RIA. Shown are plasma from four male subjects (clockwise from top left) subject 1 (triglycerides, 152 mg/dl, cholesterol, 201 mg/dl), subject 2 (triglycerides, 81 mg/dl, cholesterol, 128 mg/dl), subject 3 (triglyceride, 68 mg/dl, cholesterol, 170 mg/dl), and subject 4 (triglyceride, 97 mg/dl, cholesterol, 210 mg/dl). Fractions of 1.7–2.0 ml were collected at 15-min intervals. For any single subject, equal size fractions were collected for either condition A, B, or C. Elution volume of LDL (d 1.020–1.050 g/ml), HDL (1.063–1.21 g/ml), and bovine serum albumin are indicated.

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TABLE 1. Distribution of apoA-IV

Incubation Conditions[a]	VLDL + LDL	HDL	Lipoprotein Free
		%	
4°C	1.3 ± 0.7	4.8 ± 1.7	93.8 ± 2.3
37°C (− DTNB)	6.7 ± 2.6[b,c]	28.3 ± 6.5[b,c]	65.0 ± 5.7[b,c]
37°C (+ DTNB)	1.6 ± 0.3	3.2 ± 0.5	95.2 ± 0.7

[a]Tabulation of data ± SEM from the four subjects shown in Fig. 1.
[b]Significantly different from unincubated 4°C controls.
[c]Significantly different from 37°C + DTNB incubations.

was in the free protein region (**Table 1**). All of the apoA-I eluted in a single peak associated with HDL-containing fractions, and most (60.8 ± 6.4%) of the apoE was associated with HDL_2 present in the ascending portion of the apoA-I peak, with the remaining apoE (39.2 ± 6.4%) eluting with subfractions of VLDL at the void volume of the column and over the elution volumes of VLDL + LDL.

With 6 hr incubation at 37°C, the cholesteryl ester content of plasma was increased (**Fig. 2**), and an increased amount of apoA-IV became associated with VLDL + LDL-containing and HDL-containing fractions (B panels of Fig. 1 and Table 1). There was no apparent change in the distribution of apoA-I. ApoA-IV in HDL was associated with the larger HDL particles (HDL_2), coeluting with apoE in the HDL fractions. During incubation there were losses of apoE from HDL to the lighter lipoprotein fractions (B panels of Fig. 1 and **Table 2**).

The inclusion of the LCAT inhibitor DTNB (1 mM) during incubations completely blocked the esterification of free cholesterol (Fig. 2). Compared to unincubated controls, DTNB incubation resulted in no change in the amount of VLDL + LDL apoA-IV and an insignificant small reduction in the amount of HDL-associated apoA-IV (panel C, Fig. 1 and Table 1). During DTNB incubations, apoE from HDL shifted to the lighter lipoprotein fractions to the same extent as in samples incubated in the absence of DTNB (Fig. 1 and Table 2). In subjects 1 through 4, the apoA-IV levels were, 29, 45, 32, and 40 mg/dl and apoA-I levels were 128, 143, 103, and 138 mg/dl, respectively. Thus, the average mass ratio of apoA-I to apoA-IV was 3.5.

Gel filtration of plasma provided sufficient resolution to demonstrate the redistribution of apoA-IV to HDL subclasses resulting from LCAT action, but it was inadequate to demonstrate changes in HDL particle sizes. We, therefore, examined plasma samples, using gradient gel electrophoresis and immunolocalization of apoA-I. Duplicate samples of subject 1 plasma were incubated in the absence and presence of 1 mM DTNB. Following isolation of d 1.006–1.25 g/ml fractions, samples were subjected to 4–30% gradient gel electrophoresis and stained for protein, lipid, and immunolocalization of apoA-I by im-

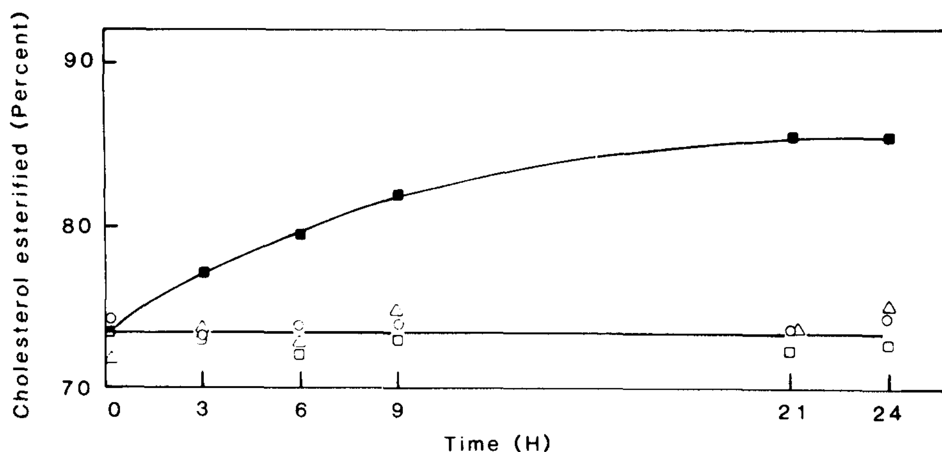

Fig. 2. Effect of DTNB or heat on esterification of cholesterol in plasma. Plasma was incubated at 4°C (○) or 37°C in the absence (■) or presence (□) of 1 mM DTNB. Additional plasma samples were preincubated at 56°C for 30 min prior to incubation at 37°C in the absence of DTNB (△). Triplicate aliquots (50 μl) were removed at the indicated times for determination of free and total cholesterol by enzymatic assay. Esterified cholesterol was determined by the difference. Data points represent the average from two normolipidemic subjects (subjects 1 and 3).

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TABLE 2. Distribution of apoE

Incubation Conditions[a]	VLDL-LDL	HDL
	%	
4°C	39.2 ± 6.4	60.8 ± 6.4
37°C (- DTNB)	48.7 ± 9.1[b]	51.3 ± 9.1[b]
37°C (+ DTNB)	49.9 ± 9.2[b]	50.1 ± 9.2[b]

[a] Tabulation of data ± SEM from the four subjects shown in Fig. 1.
[b] Significantly different from unincubated 4°C controls.

munoblot analysis (**Fig. 3**). Additional aliquots were analyzed by isopycnic density gradient ultracentrifugation for determination of apoA-I distribution (**Fig. 4**). ApoA-I was used as an HDL marker since apoA-IV is easily displaced from HDL by ultracentrifugation or gradient gel electrophoresis (12). Incubation at 37°C resulted in a shift from HDL_3 to HDL_2 staining intensity of lipid, protein, and apoA-I (Fig. 3). Incubation in the presence of DTNB increased the staining intensity of HDL particles intermediate in size between HDL_2 and HDL_3. Isopycnic density gradient centrifugation of incubated plasma demonstrated a shift in the distribution of apoA-I from particles having an average density of 1.140 g/ml in the unincubated control (Fig. 4A) to a density of 1.115 g/ml after incubation at 37°C in the absence of DTNB (Fig. 4B), verifying the findings on gradient gel electrophoresis. Incubation in the presence of DTNB (Fig. 4C) resulted in only a minor shift in the distribution of apoA-I to a density of 1.132 g/ml. The percentages of apoA-I recovered in the d 1.006–1.25 g/ml fraction following the initial isolation by ultracentrifugation were 81.2%, 87%, and 77.5% for unincubated, incubated, and DTNB-incubated plasma, respectively. Additional losses of apoA-I from HDL during the subsequent isopycnic density gradient ultracentrifugation were 19.6%, 18.6%, and 16.2% (recovered in the bottom two fractions), for unincubated, incubated, and DTNB-incubated plasma, respectively. It is not known which specific lipoprotein subfractions lose apoA-I during ultracentrifugation. In contrast, as noted above, after gel filtration of plasma, all apoA-I was recovered in a single broad peak corresponding to the particle size spectrum of HDL; no distinct apoA-I peak was found in the lipoprotein-free fractions.

In order to determine the adequacy of the DTNB control, experiments were designed to determine whether DTNB can affect lipoprotein distribution of apoA-IV by mechanisms independent of LCAT inhibition. Another sample of fresh human plasma (subject 1) was incubated without DTNB for 6 hr at 37°C and was either *1*) gel-filtered immediately in the presence and absence of DTNB (**Fig. 5A**) or *2*) stored for 48 hr at 4°C in the presence and absence of DTNB prior to gel filtration (Fig. 5B). As shown in Fig. 5A, 41% of apoA-IV was contained within fractions that normally contain lipoproteins (fractions 22–38) with the remainder in the free protein region. The addition of DTNB just prior to gel filtration had essentially no effect on the distribution of apoA-IV. However, storage of plasma for 48 hr caused a marked reduction of the amount of apoA-IV associated with lipoprotein-containing fractions regardless of whether DTNB was present during storage (Fig. 5B). However, storage of a gel-filtered HDL fraction obtained from plasma incubated in the absence of DTNB resulted

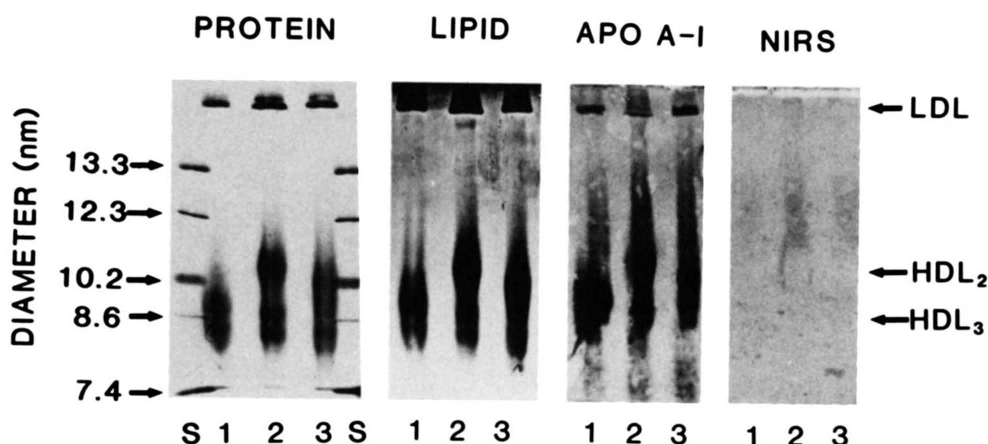

Fig. 3. Comparison of HDL-size particles following incubation of plasma (subject 1) in the absence and presence of DTNB. Density fractions of 1.006 < d < 1.25 g/ml were isolated from unincubated whole plasma (lane 1) or from plasmas incubated at 37°C for 6 hr in the absence (lane 2) and presence (lane 3) of 1 mM DTNB. Fractions were concentrated and dialyzed into gradient gel buffer, and applied to 4–30% gradient gels and electrophoresis was performed as described in Materials and Methods. Gels were subsequently stained for protein, lipid, or transferred to cellulose nitrate paper for immunological identification of apoA-I and nonspecific staining with nonimmune rabbit serum (NIRS). High molecular weight protein standards (Pharmacia) of thyroglobulin, 660 K, diameter, 13.3 nm; apoferritin, 440 K, diameter, 12.3 nm; catalase, 220 K, diameter, 10.3 nm; lactic dehydrogenase, 140 K, diameter, 8.6 nm; and albumin, 67 K, diameter, 7.4 nm were applied to lane S.

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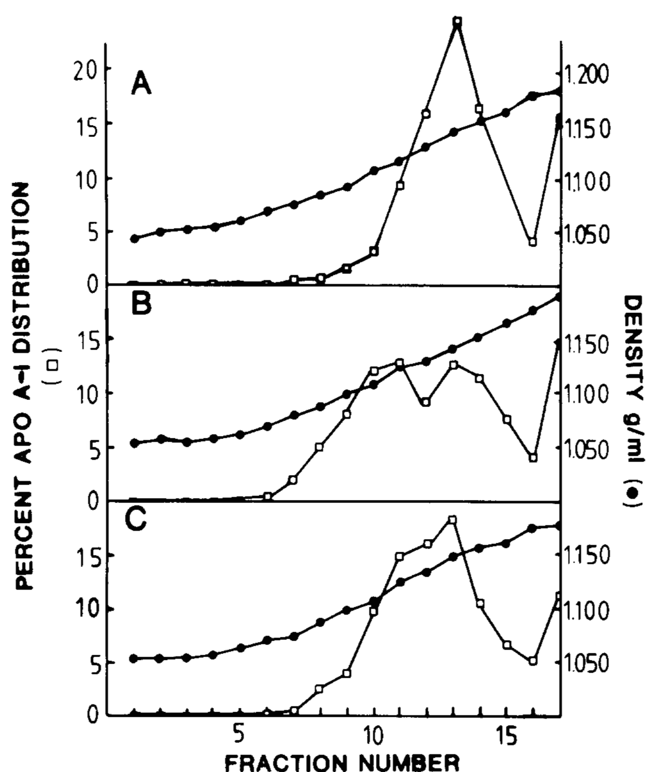

Fig. 4. Comparison of HDL density following incubation of plasma (subject 1) at 37°C in the absence and presence of DTNB. The 1.006 < d < 1.25 g/ml fractions (Fig. 3) were also fractionated by isopycnic density gradient centrifugation as described in Materials and Methods. NaBr density (●) was monitored by refractometry and apoA-I mass (□) by RIA; unincubated plasma (A), plasma incubated in the absence (B) and presence (C) of DTNB.

in minimal loss of apoA-IV from HDL to the free protein region upon rechromatography (**Fig. 6**).

To determine whether the distribution of apoA-IV was dependent on LCAT, studies were conducted on plasma samples that were preheated to 56°C for 30 min to irreversibly inactivate the enzyme (see Fig. 2). Heated plasma was then immediately gel-filtered or incubated for an additional 6 hr at 37°C prior to gel filtration. An identical apoA-IV distribution was observed in preheated plasma that was either immediately gel-filtered (not shown) or subsequently incubated for 6 hr at 37°C (**Fig. 7**). However, when plasma was preincubated at 4°C instead of at 56°C and then incubated for an additional 6 hr at 37°C, a marked amount of apoA-IV associated with lipoproteins (Fig. 7).

To determine whether LCAT played a direct role in movement of apoA-IV onto lipoproteins, LCAT was purified from human plasma and added back to LCAT-inactivated (i.e., preheated) plasma. In order to estimate the amount of LCAT to add to heated plasma and to determine whether the addition of 2-mercaptoethanol (45, 46) was a necessary addition for incubation studies, plasma samples were prelabeled with [4-^{14}C]cholesterol at 4°C. An aliquot was removed and the remainder was heated to 56°C for 30 min. Preheated plasma samples (50 μl) were then incubated for 0, 6, or 24 hr at 37°C in the presence or absence of 2-mercaptoethanol (5 mM) with no or increasing concentrations of purified LCAT in a total volume of 260 μl in 50 mM phosphate-buffered saline, pH 7.4. Control plasmas that were not preheated were incubated for 0, 6, or 24 hr at 37°C. The amount of [4-^{14}C]cholesterol esterified was determined as described in Materials and Methods. As shown in **Fig. 8,** LCAT addition was necessary for cholesterol esterification in this system, and an equivalent amount of enzyme activity was observed in either the presence or absence of 2-mercaptoethanol. From these data it was determined that approximately 1.1 μg of enzyme per 50 μl of heated plasma was necessary to reconstitute the normal level of LCAT activity observed in unheated plasma. To determine whether LCAT addition caused an alteration of the apoA-IV distribution, either active (unheated) or inactive (heated) LCAT (11.7 μg) was added to heat-inactivated plasma (500 μl) from subject

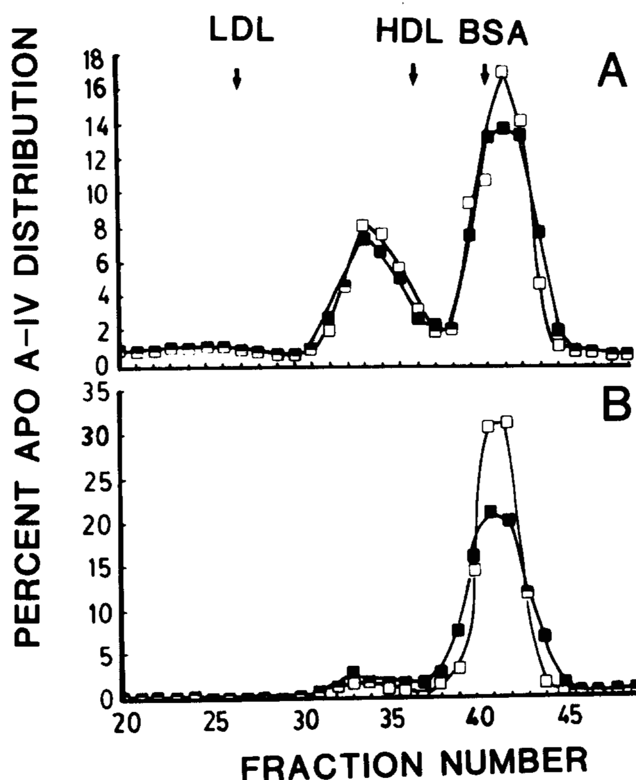

Fig. 5. Effect of DTNB and storage on gel filtration distribution profile of apoA-IV in incubated human plasma. Human plasma was incubated for 6 hr at 37°C and immediately gel-filtered on Bio-Gel A-5m in the absence and presence of 1 mM DTNB (A) or stored for 48 hr at 4°C in the absence and presence of 1 mM DTNB prior to gel filtration without further additions (B). With DTNB, (□); without DTNB, (■). Elution volume of LDL (d 1.020–1.050 g/ml), HDL (1.063–1.21 g/ml), and bovine serum albumin are indicated.

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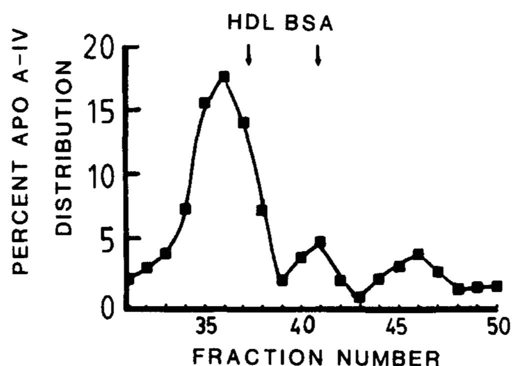

Fig. 6. Gel filtration of apoA-IV-rich fraction of HDL. Fraction 35 from subject 1 in Fig. 1B (whole plasma incubated without DTNB) was stored for 5 days at 4°C and applied to a gel filtration column. Fractions were monitored for apoA-IV. Elution volume of HDL (1.063-1.21 g/ml) and bovine serum albumin are indicated.

#4. Plasma samples were incubated for 6 hr or 24 hr at 37°C (without 2-mercaptoethanol) prior to gel filtration. **Fig. 9** shows the gel-filtered distribution of apoA-IV in this plasma. When plasma was incubated for 6 hr with active LCAT, a marked shift in lipoprotein-associated apoA-IV was observed. Following 24 hr incubation, essentially all the apoA-IV that normally elutes with free protein region was associated with lipoproteins. The addition of heat-inactivated LCAT had no effect on the apoA-IV distribution.

DISCUSSION

In the present report, we have documented that LCAT activity influences the distribution of apoA-IV among human lipoproteins. Incubation of whole human plasma under conditions in which LCAT is active results in a shift of lipoprotein-free apoA-IV onto the plasma lipoproteins. Prior inactivation of LCAT blocks this shift. The addition of a highly purified preparation of LCAT to plasma in which the enzyme had been inactivated restores the shift of apoA-IV onto the lipoproteins. Thus, the effect specifically depends on the action of LCAT. The findings of the present study are consistent with prior work (19) demonstrating an LCAT-dependent shift of free apoA-IV onto lipoproteins in the plasma of fasting rats. In addition, we demonstrated an LCAT-independent shift of apoE and a stable association of apoA-I with HDL.

Studies of Quarfordt, Boston, and Hilderman (47) and Chajek and Fielding (48) demonstrate that plasma lipid transfer proteins produce an equimolar exchange of VLDL triglyceride for HDL cholesteryl ester. This would result in an increased HDL core volume, because a mole of triglyceride occupies 1.5 times the volume of a mole of cholesteryl ester (49). Our result that HDL particles enlarge even when cholesterol esterification was inhibited could be the result of core lipid transfer events that occur between particles, and are consistent with studies of Hopkins, Chang, and Barter (50) and Nichols, Gong, and Blanche (46). In the studies of Hopkins et al. (50), when HDL were incubated with VLDL or Intralipid in the absence of LCAT activity and without addition of lipoprotein lipase, HDL_3 enlarged and became triglyceride-enriched, and small particles formed. Similar enlargement of HDL_3 and formation of small lipoprotein particles were described in the study of Nichols et al. (46), when human plasma was incubated with two types of LCAT inhibitors.

The fact that the HDL core expands whether or not LCAT is active, but free apoA-IV binds only when LCAT is active suggests that particle core enlargement is not the only factor responsible for the shift of apoA-IV from the lipoprotein-free fractions to HDL. Since incubation with active LCAT partially depletes the lipoprotein phospholipid and cholesterol substrates, "gaps" in the lipoprotein surface could form. The increased volume of cholesteryl ester in the lipoprotein core further contributes to these "gaps." The dependence of apoA-IV binding on LCAT activity supports the hypothesis that "gaps" are the sites on the lipoprotein surfaces to which free apoA-IV binds (cf. 19). ApoA-IV may have a special role in human plasma since, unlike other apolipoproteins, it is largely free. Thus, it may provide a reservoir of amphipathic protein to stabilize lipoprotein particles as they enlarge under the influence of LCAT.

Our control studies (Figs. 5 and 6) suggest that human apoA-IV is loosely bound to lipoproteins. The relatively loose binding of apoA-IV to lipoproteins suggests that this protein is in a dynamic equilibrium with other plasma components. Possibly, phospholipid, cholesterol, and/or

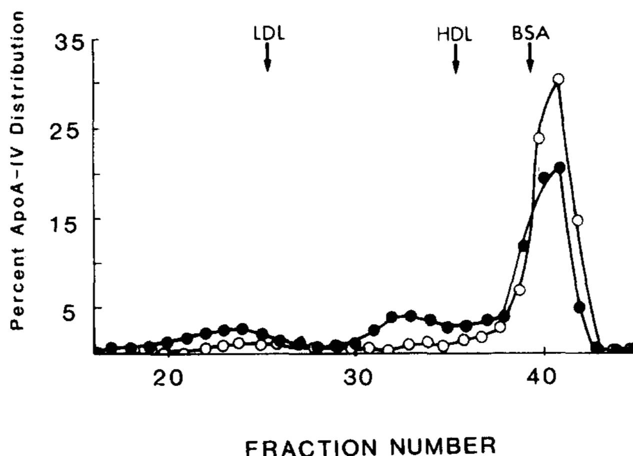

Fig. 7. Effect of heat-inactivation of plasma LCAT on distribution of apoA-IV. Plasma (subject 1) was either preincubated at 4°C (●) or 56°C (○) for 30 min prior to incubation for 6 hr at 37°C. Plasmas were gel-filtered on Bio-Gel A-5m and aliquots of fractions were assayed by RIA for apoA-IV content.

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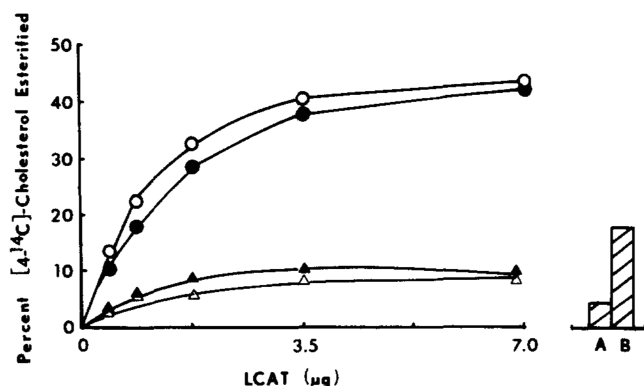

Fig. 8. Effect of LCAT on esterification of [4-^{14}C]cholesterol-prelabeled heated plasma. [4-^{14}C] cholesterol (10 μCi) in 8 μl of ethanol was forcefully injected into 2 ml of plasma and incubated for 1 hr at 4°C. Aliquots were removed for (A) control (6 hr) or (B) control (24 hr) incubations at 37°C (striped boxes). The remaining plasma was heated to 56°C for 30 min and then 50-μl aliquots were incubated with purified LCAT for 6 hr (△, ▲) or 24 hr (○, ●) in the absence (open symbols) or presence (solid symbols) of 2-mercaptoethanol (5 mM) in a total volume of 260 μl. Aliquots were extracted as indicated in Materials and Methods for determination of percent [4-^{14}C]cholesterol esterified.

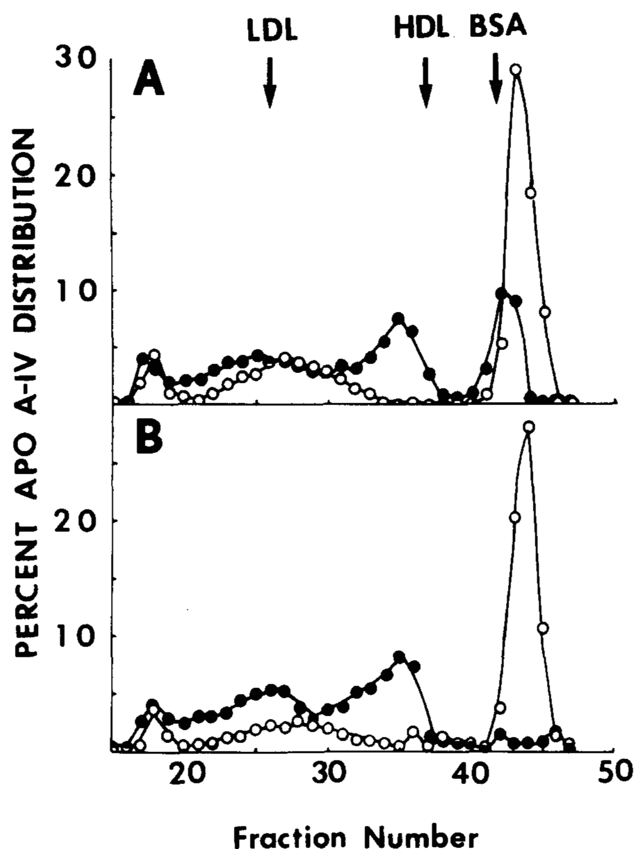

Fig. 9. Effect of LCAT on the distribution of apoA-IV in heated plasma. Plasma from subject 4 was preincubated at 56°C for 30 min and then plasma fractions (0.5 ml) were incubated for 6 hr (A) or 24 hr (B) at 37°C in the presence of heat-inactivated (○) or active (●) LCAT (11.7 μg of protein) in a total volume of 1 ml (500 μl plasma + 450 μl inactive or active LCAT fraction + 50 μl of 10× concentrated phosphate-buffered saline). Plasmas were gel-filtered on Bio-Gel A-5m and aliquots of fractions were assayed by RIA for apoA-IV content.

other apolipoproteins exchange with apoA-IV in competition for sites on the surface of LCAT-modified lipoproteins. In vivo, this effect may be especially pronounced due to constant influx of competitors from plasma and tissue sources. In rats (19), incubation of plasma under conditions in which LCAT was inactivated with DTNB similarly resulted in displacement of lipoprotein-associated apoA-IV. We observed a similar trend in the four fasting human plasma samples; however, the drop in lipoprotein-associated apoA-IV was not statistically significant. In unincubated plasma of one fasting subject, 9.6% of apoA-IV was associated with HDL; following incubation with DTNB, only 4.0% was HDL-associated.

ApoA-IV is an activator of LCAT, and is a better activator than apoA-I when saturated phosphatidylcholine is used as the fatty acyl donor (20). Based on these earlier studies (20) and our own, we propose that apoA-IV may have a unique physiologic role in the LCAT reaction. By filling "gaps" in the lipoprotein surface, apoA-IV may allow utilization of those residual phosphatidylcholine molecules (the saturated ones) for which LCAT has a lower activity. Thus, by filling "gaps," apoA-IV may allow better access of LCAT to its substrates, and may allow further depletion of surface elements, and creation of additional cholesteryl ester.

In addition, apoA-IV may facilitate the LCAT reaction by providing additional phospholipid and cholesterol substrate to HDL. These lipids may be present in small phospholipid-enriched lipoprotein particles that contain apoA-IV, as demonstrated by affinity chromatography of the d > 1.21 g/ml fraction of plasma (16). The coelution of a particle containing apoA-I, apoA-IV, and lipid in the size range of small HDL has been observed in the descending portion of the HDL peak of gel-filtered human plasma (12). Whether or not this is the same particle isolated from the d > 1.21 g/ml fraction as described by Ohta, Fidge, and Nestel (16) is uncertain. Similar small apoA-IV lipoprotein particles have been described in rat mesenteric lymph; however, these particles have a density of 1.15–1.18 g/ml (51). In contrast, however, the studies of Weinberg and Spector (52) suggest that most apoA-IV in human plasma circulates as a dimer unassociated with lipid.

Lastly, our finding that apoA-IV is largely present on human HDL_2 following incubation in vitro, while it is not normally found on plasma HDL_2 in vivo, suggests that apoA-IV-rich HDL_2 could represent a unique subpopulation that is cleared rapidly from the circulation. Although specific receptors for apoA-IV have not been described in humans, they have been reported to be present in rat hepatocytes (53). The presence of similar receptors in

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human liver would lend further support to the speculation that apoA-IV plays a key role in the clearance of HDL_2 from human plasma.

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